MANGALORE UNIVERSITY

Screening of Potential Natural Bioactive Compounds from Some Plant Extracts and their Effects on Malaria Transmitting Mosquitoes

Final Ph.D. thesis synopsis

Submitted

To

Department of Applied Botany

Mangalore University

By

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(Reg No. MU/ACC /Ph.D./CR-52/2015-16/A3, 10 February 2016)

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2023

INTRODUCTION

Infectious diseases spread by mosquitoes have been a problem around the world for eons. Mosquito populations are the primary vector for many of the world's most deadly vector-borne diseases. They are bothersome pests and the main carriers of tropical diseases. Compared to other arthropod groups, it can spread more diseases and infect millions of people worldwide (1). More than 17% of all infectious diseases are vector-borne, and they are responsible for more than 700,000 fatalities each year. Malaria, lymphatic filariasis, dengue, chikungunya, rift valley fever, yellow fever, zika, Japanese encephalitis, West Nile fever, and many other deadly diseases are spread by mosquitoes, the most common of which belong to the Anopheles, Aedes, and Culex genera. These vectors introduce pathogens on a daily basis into countries where they were previously unknown (2).

Malaria is an infectious disease transmitted by mosquitoes that remains a leading cause of morbidity and mortality on a global and regional scale. There were an estimated 241 million cases of malaria in 2020, up from 227 million in 2019, and over 627,000 deaths. Across Africa's tropical regions, children are more susceptible to malaria than adults (3). The Global Malaria Program (GMP) is an initiative of the World Health Organization (WHO) with the goal of eradicating malaria worldwide by 2030 (4). According to the Central Bureau of Health Education (CBHE) and the Directorate of the National Malaria Eradication Programme (NMEP), malaria remains the most important vector-borne disease in India. In 2020, the National vector-borne disease control programme (NVBDCP) reported 29340 malaria cases and two deaths (5). It is critical to control the mosquito population in order to reduce the transmission of infectious malaria and achieve the GMP goal.

Mosquitoes belong to the Culicidae family, which contains over 3,500 different species and subspecies. Malaria is a deadly disease caused by active female Anopheline bloodsuckers. Warm temperatures are required for these mosquitos to breed and spread the disease (6, 7). Of the 53 anopheline species present in India, nine are vectors of malaria. The efficient "type form" of *Anopheles stephensi* Liston is known to be a major urban malaria vector in the Indian subcontinent and the Middle East (7). Rapid urbanisation in developing countries increases the population of the endophilic and endophagic mosquito *An. stephensi*. The WHO considers *An. stephensi* to be a highly competent vector of *Plasmodium falciparum* and *Plasmodium vivax* (8), and its rapid spread in urban areas threatens malaria elimination. Because most vector-borne diseases lack vaccines

and other effective prevention methods, as well as because of the complexity of disease life cycles, highly integrated approaches that target the disease transmission system rather than just the pathogens are required (5). However, disease control is now jeopardised by rapidly increasing insecticide resistance in insect populations, emphasising the importance of launching specific strategies to reduce vector-mediated transmission (9).

Technical advances in the 1930s and 1940s, such as the introduction of DDT and other insecticides, resulted in the apparent eradication of the disease in the 1950s and 1960s (10, 11, and 12). Although effective, the repeated use of synthetic insecticides has disrupted natural biological control systems and, in some cases, resulted in widespread physiological resistance (13). These issues have necessitated the development of alternative strategies based on environmentally friendly products. These ever-increasing issues demand an intensive search for new products that are environmentally safe, target-specific, and biodegradable (14).

Plants are abundant in bioactive molecules, which are extremely useful in daily life. Historically, ancient people used botanical preparations as insecticides (21). The biologically active chemicals have the potential to be a source of mosquito control agents (35). As a result, plant exploration will help the human population overcome the challenges of an integrated vector control strategy, sustain malaria elimination, and prevent its reintroduction. Plant extracts are less harmful to non-target organisms, such as humans (36). There have been reports of medicinal plants from the families Annonaceae, Papilionaceae, Meliaceae, Mimosaceae, and Lamiaceae with insecticidal activity, including ovicidal, oviditerrent, larvicidal, pupicidal, antifeedant, and repellent properties (35). Alkaloids, tannins, terpenoids, steroids, and saponins, among other phytochemicals, were effective as larvicidal, ovicidal, pupicidal, and insecticidal agents (46).

It is critical to investigate the toxicity level of botanical preparations before testing them in the environment. Cytotoxicity studies are a useful preliminary step in determining the potential toxicity of a test substance, particularly plant extracts or physiologically active chemicals derived from plants. Cellular toxicity studies are important in this context because they teach us how to make insecticides with little to no toxicity. The MTT assay (using the NIH-3T3 cell line) is the preliminary method for studying cytotoxicity. Such research is required to determine whether the biological activity is a distinct entity or should be classified as general toxicology (47, 48). It is widely acknowledged that using medicinal plants without first determining their efficacy and

safety profile may have negative effects on several organs. So, an oral acute toxicity study will help figure out how dangerous the extract is (49).

The current scenario for mosquito control is based on the use of target- and stage-specific, cost-effective, and biodegradable phytoproducts. This has necessitated ongoing efforts to find and develop environmentally safe, biodegradable, and low-cost mosquitocides. The present study deals with a screening laravicidal study of *Pajanelia longifolia*, *Psidium gujava*, *Aegle marmelos*, *Couroupita guianensis*, and *Sphagneticola trilobata*. Furthermore, the insect susceptibility of selected plant *P. longifolia* leaf extract was evaluated, including ovicidal, oviditerrence, fecundity, larvicidal, pupicidal, growth index, and repellence activity. Also, the anti-plasmodial activity, chemical makeup, histology, cytotoxicity, and acute oral toxicity of some plant extracts were looked at.

AIM AND OBJECTIVES

Aim:

- The present research work focused on the larvicidal activity of different plant extracts and the effect of selected plant extracts on different stages of *An. stephensi* mosquitoes.

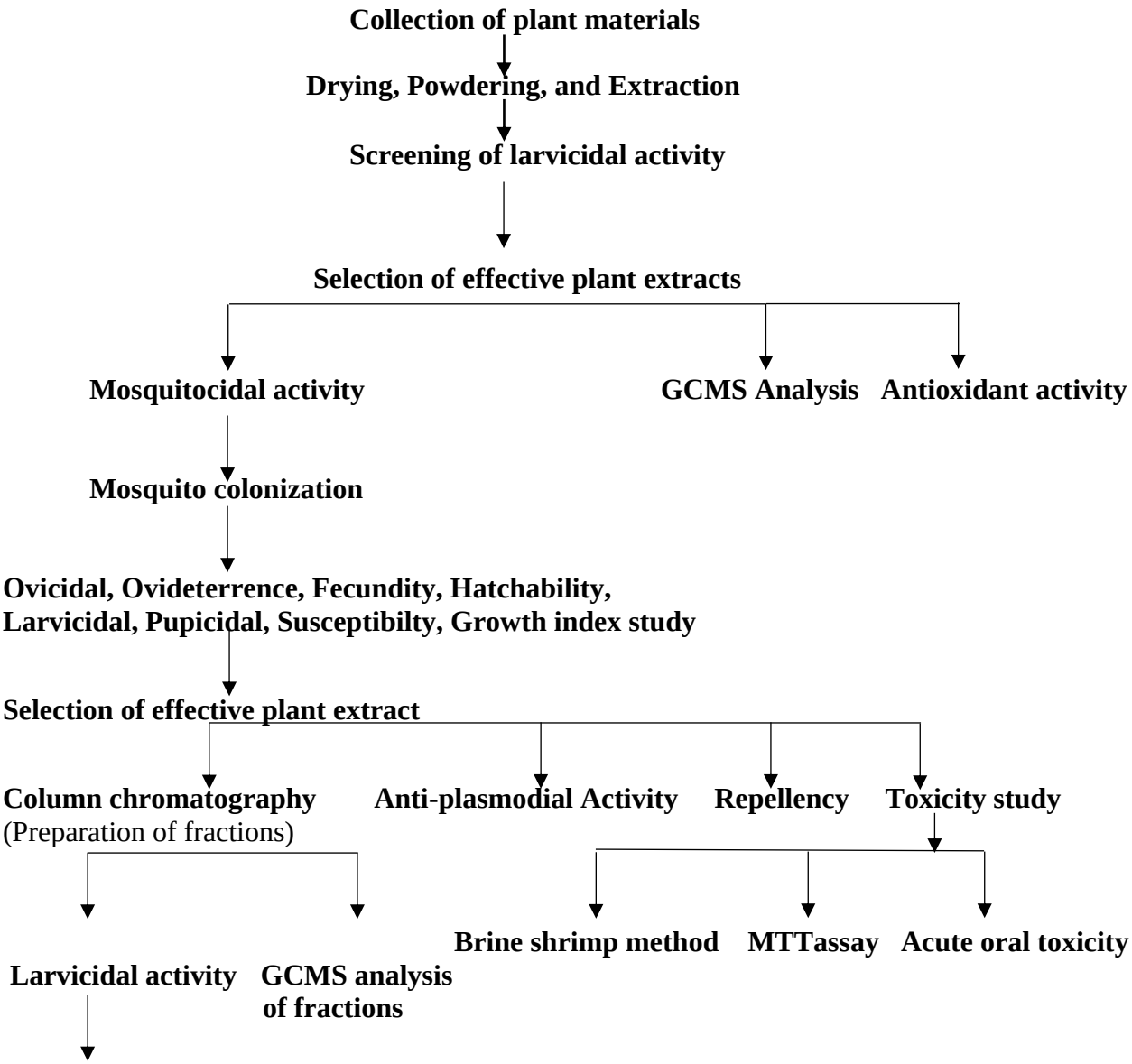
Objectives:

- To assess the larvicidal potential of different solvent extracts of selected plants. To evaluate lethal concentrations (LC_{50} and LC_{90}) of the extracts.
- Evaluation of primary phytochemicals, antioxidant activities of *P. longifolia* leaf extracts.
- GC-MS analysis of effective plant extracts.
- To evaluate the selected extracts on different instars of *An. stephensi* larvae.
- To evaluate ovicidal, Ovi-position, fecundity, hatchability, pupicidal activity, susceptibility, and growth index of selected extracts.
- To evaluate *in-vitro* toxicity of selected plant extracts.
- To evaluate the anti-plasmodial activity of the selected extract.
- To evaluate oral acute toxicity of *P. longifolia* methanol leaf extract on Wistar albino rats.
- To evaluate the repellency activity of the selected extract.
- To separate the fractions from the selected plant extract and study the larvicidal activity.

❖ GC-MS analysis of the effective fraction.

SUMMARY OF THE WORK DONE

- **Schematic representation of research work:**



1. The collection of plant materials from different locations of Kasaragod, Kerala.
2. A Large number of mosquitoes were colonised for further study and studied the effect of temperature and humidity during the mosquito colonization at NIMR, Bangalore.
3. Preparation of solvent and aqueous extracts from selected plants and their preliminary study

on larvicidal activity against *An. stephensi.*

4. The lethal concentrations (LC_{50} and LC_{90}) of each extract were evaluated and a more potent plant was used for further analysis.
5. The larvicidal active plant extracts were primarily screened for phytoconstituents and characterized for Gas chromatography-mass spectrometry (GC-MS).
6. The potent plant extracts were screened on different instars of *An. stephensi*. In addition, the extracts were screened for ovicidal, fecundity, hatchability, oviposition, pupicidal activities.
7. The potent plant extracts were screened for mosquito growth index study.
8. The effective plant extracts were screened for mosquito susceptibility test (cone method).
9. The effective plant extract screened for repellency activity.
10. Larvicidal potent extract was assessed for brine shrimp toxicity assay and *in-vitro* cytotoxicity study.
11. Antimalarial property was studied by *in-vitro* anti-plasmodial activity.
12. The larvicidally potent extract was assessed for acute oral toxicity using animal model by monitoring behavioral, biochemical and histological modifications
13. The Column chromatography of potent extract were carried out and separated fractions.
14. The larvicidal activity of fractions are carried out and lethal concentrations (LC_{50} and LC_{90}) of each fractions were evaluated and more potent fraction was used for further analysis.
15. The histopathological changes were evaluated to check the effect of extracts on *An. stephensi* larvae.
16. Further GCMS analysis was carried out for the potent fraction.

CHAPTER 1: INTRODUCTION

This chapter contains a crucial introduction to the thesis for our investigation. The first portion covers the introduction of mosquito vectors, distribution, control methods, synthetic chemicals, biological control, and natural products. The use of medicinal plants in vector biology and their effects on all developmental stages of mosquito development and life cycles, with a note

on plant extract phytochemical study methods, continued with a brief introduction to the anti-plasmodial activity, and we also discussed a study of plant extract against non-target species. A brief introduction to cell viability and acute oral toxicity were carried out.

CHAPTER 2: REVIEW OF LITERATURE

This chapter discusses the recently published literature reports that were relevant to the current study. The previous studies of research plants are reviewed in detail. Mosquito identification, distribution, synthetic chemicals, natural products, ovicidal activity, ovi-position, fecundity, hatchability, larvicidal activity, pupicidal activity, susceptibility, repellency, and the growth index of the most recently reviewed articles were also covered in the chapter. Further, the antiplasodial activity methods and the acute oral toxicity study methods were also reviewed in detail.

CHAPTER 3: MATERIALS AND METHODS

Instruments and chemicals (reagents and solvents) used for research were reported. Plant identification and collection, extract preparation, and screening for larvicidal activity helped to select the most effective plant extracts for further study. However, plant extracts were chosen based on their qualitative, quantitative, antioxidant, ovicidal, ovi-position, fecundity, hatchability, larvicidal, pupicidal activity, susceptibility, repellency, growth index, and non-target species. In addition, the cell viability and oral acute toxicity assays were carried out according to OECD - 425 Guidelines on Wistar albino rats. The anti-plasodial activity of a specific plant extract was tested.

The LC_{50} and LC_{90} values were calculated using the log-probit model using SPSS software, and Tukey's one-way ANOVA was used to measure mean mortality and standard error mean with the help of SPSS 22; $p<0.05$considered statistically significant.

CHAPTER 4: RESULTS

The results were compiled in the fourth chapter to support the thesis's objectives. The plant based vector control is the best approach in minimising the mosquito population thereby inhibiting the transmission of the disease. The preliminary screening method is an effective way to assess the potential plant for larvicidal activity and the results showed five plants as active for larvicidal activity and methanol extract as a suitable solvent for extracting more number of compounds from

the plants. The highest larvicidal activity (100%) was registered by *P. longifolia* leaf extracts. In 24 hours, the most potent methanol extract of *P. longifolia* showed 100% mortality at 1050 ppm, with LC_{50} and LC_{90} values of 446.56 and 750.65 ppm, respectively. With increasing exposure time, the mortality rate rises. These values are clearly observed after 48 hours of exposure at 950 ppm with LC_{50} and LC_{90} values of 453.94 and 685.02 ppm, respectively. Petroleum ether and chloroform extracts demonstrated minimal activity, with LC_{50} values of 2458.72 and 2118.56 ppm at 24 and 48 hours, respectively, and LC_{90} values of 7653.88 and 7536.79 ppm at 24 and 48 hours. The findings clearly show that mortality increases with time spent exposed to leaf extracts.

The crude leaf extract percentage yield ranged from 9.4 to 32.4 gm. It was discovered that methanol extract (16.2 gm) had the highest yield, followed by chloroform (9.2 gm) and petroleum ether (4.7 gm) extracts (Table 1). The preliminary qualitative phytochemical analysis reveals that the extracts of *P. longifolia* contain a wide range of phytochemical constituents, including alkaloids, carbohydrates, starch, glycosides, saponins, steroids, phenols, tannins, flavonoids, proteins, and resins.

Antioxidant activity was carried out by free radical scavenging method and reducing power assay. In the present study methanol extract of *P. longifolia* showed potential free radical scavenging activity with an increase in concentration. The assay was assessed by a drop in absorbance at 517 nm, which indicated the antioxidant's ability to reduce. The IC_{50} value of methanol extract is 90.43 µg/mL and the standard is 54.27 µg/mL. The results may indicate that the radical scavenging capacity of the extract might be related to its concentration of phytochemicals. The reducing capability of an extract can be monitored by the formation of blue colour at 700 nm. The reducing power assay was computed using a regression equation calibration curve, i.e. $y = 0.0003x + 0.0149$ with R^2 of 0.9849 and expressed as ascorbic acid equivalents. The activity was seen increasing with an increase in the concentration.

The oviposition deterrent activity of *P. longifolia* extracts differed. According to the findings, the methanol leaf extract is the most effective oviposition deterrent, followed by chloroform and petroleum ether in decreasing order of efficacy. *An. stephensi* oval susceptibility of methanol leaf extract exhibited 100% mortality at 900 ppm, with LC_{50} and LC_{90} values of 116.06 ppm and 445.79 ppm, respectively. At higher concentrations, petroleum ether and chloroform extracts were lethal. Further, the larval mortality of different instars were observed after treatment with *P. longifolia* leaf extracts. According to the findings, the 1^{st} instars were

more vulnerable than the 2nd and 3rd instars. The percent mortality of pupae in comparison petroleum ether and chloroform extract, methanol extract were the highest pupal mortality. With increasing exposure time, the mortality rate rises.

The fecundity, hatchability, and growth index of three extracts were studied and methanol extract found to be more effective when compared to the other two extracts. Among extracts, toxic nature was exhibited by methanol extract followed by chloroform and petroleum ether extract against brine shrimp which was a non-target (Brine shrimp) organism. The GC-MS analysis results revealed a variety of phytoconstituents in all crude extracts which are effective against vector control. The effective methanol extract was selected for the further studies. The toxicity of methanol extract was studied by *in-vitro* cytotoxicity (MTTassay and acute oral toxicity). The cells viability (MTT assay using NIH-3T3 cell-line) was altered due to the effect of extracts. The acute oral toxicity result confirmed that the extract LD_{50} was greater than 2000mg/kg of body weight. No adverse effect was noticed in the oral acute toxicity on Wistar albino rats.

The repellency activity of methanol extract was carried out and exhibited low repellency activity against the methanol extract. The anti-plasmodial activity of the same extract also reported minimum to least activity against *Plasmodium falciparum*. Further, the extracts were subjected to column chromatography and about 9 fractions were separated and larvicidal activity was carried out. The 5th fraction (Fraction B) showed 100% larval mortality. The morphology and histology of the moribund larvae show the effect of the fraction. The GC-MS results of the fraction revealed the presence of phytoconstituents. This result highlights the use of plant extracts of *P. longifolia* as an effective agent against the developmental stages and lifecycle of malaria-transmitting *An. stephensi* mosquitoes.

CHAPTER 5: DISCUSSION

The complicated disease of human malaria is influenced by the interactions between the Plasmodium species, the parasite carried by the Anopheles mosquito, humans, and the environment. More people have been impacted by mosquitoes than any other insect in many developing nations, primarily in poor communities, with cumulative impacts on the population as a whole, placing a socioeconomic burden on humanity (15, 16). In India, due to favorable ecological conditions, mosquito-borne diseases are nearly ubiquitous in almost the entire country (17).

The management of a growing mosquito population is a significant global public health

challenge because there are no viable malaria vaccines. Vector control is a key component of the current global strategy to combat vector-borne diseases. At the moment, synthetic pyrethroid insecticides, organophosphates, and organochlorine pesticides are being employed as public health control measures (18, 21). Multiple insecticide resistances result from successive insecticide changes. Malaria vectors in India are resistant to DDT, malathion, and deltamethrin. The continuous usage of synthetic chemicals has led to worries about the environment and human health, as well as unwanted impacts on non-target creatures (19).

India, one of the world's top twelve countries for biodiversity, is known for its lush vegetation and abundance of medicinal plants (20). There is evidence that plants can produce a wide range of alkaloids, phenolics, oils, and secondary metabolites. These organic components have potent insecticidal capabilities, and the Romans and the Chinese utilized them in ancient times (21). Currently, pyrethrum, rotenone, neem, and essential oils are the four main categories of botanical products used to control insects. Three other categories, ryania, nicotine, and sabadilla, are also occasionally employed (22). Plant extracts are an alternative insect/mosquito management strategy for reducing the negative environmental effects of some insecticides (23, 24, and 25). In underdeveloped countries, where people have long used plants and plant parts to protect themselves from mosquitoes and where poor communities may not be able to afford synthetic insecticides, botanical insecticides can be used to their fullest potential.

Phytochemicals produced from plant sources can act as ovicidal, larvicidal, pupicidal, insect growth regulators, repellents, and oviposition attractants and can play a significant role in the interruption of the transmission of mosquito-borne diseases at the individual as well as the community level (26, 27, 28, 29, 30, 31, 32, 33, and 34). Numerous plant extracts and essential oils possess larvicidal properties against different mosquito species (14, 35, 36, and 37). Some phytochemicals are poisonous (insecticides) to mosquitoes in both their adult and larval phases, while others inhibit growth, limit reproduction, or produce olfactory stimulation, functioning as a repellent or attractant (28, 29, 31, and 32).

The late third instar larva of *An. stephensi* was the target of the current study, which assessed the larvicidal effects of *P. longifolia*, *P. gujava*, *A. marmelos*, *C. guianensis*, and *S. trilobata* leaf extracts. The overall results of the experiment demonstrated that some plant extracts were toxic to larvae, but few larvae that had been exposed to plant extracts survived to become adults. Larvicidal mortality varied with exposure and concentration. Based on fatal concentration

values across all experimental plants, *P. longifolia* was discovered to be a plant species that effectively combats *An. stephensi*.

P. longifolia (Willd.) K. Schum is an evergreen or deciduous small to medium tree that is a single species of the monotypic genus Pajanelia in the Bignoniaceae family. They can be found in deciduous and semi-evergreen mountainous rainforests of India, Sri Lanka, Myanmar, Thailand, Peninsular Malaysia, Sumatra, and Borneo, at elevations of up to 1000 metres above sea level (8). The plant is high in secondary metabolites that have medicinal value. It is well known for its medicinal properties as a hepatoprotective agent (39). In folk medicine, the young, tender leaves of the plants are used to treat nail infections and skin rashes (40). Leaf extracts containing polar components were found to have antibacterial activity (40, 41). The plant's bark powder extract contain antioxidant and antimicrobial properties (38, 42). As a result, no experimental data on the validation of *P. longifolia*'s antimosquito properties are available.

The current study intends to use *P. longifolia* leaf extracts against different developmental stages of *An. stephensi* mosquitoes. The plant methanol extract appeared the highest ovicidal deterrence activity based on percent oviposition deterrence values. The findings of this research are consistent with those of other studies published in the literature, which show a wide range of results for different plants. Kuppusamy (2008) previously assessed the oviditerrence activity of *Andrographis paniculata* Nees and found a significant difference in the number of eggs laid between the control and test groups (29). In addition, Prathibha (2014) investigated the ovideterrence activity of *Eugenia jambolana, Solidago canadensis, Euodia ridleyi*, and *Spilanthes mauritiana* leaf extracts against *An. stephensi* and found higher oviposition deterrence activity (43). The methanolic extract of *P. longifolia* showed 100% ovicidal activity. Govindarajan *et al*. (2011) studied the ovicidal activity of *Ervatamia coronaria* and *Caesalpinia pulcherrima* and reported 100% mortality at 150 ppm and 225 ppm, respectively (44). Veni *et al*. (2017) studied the ovicidal activity of *Terminalia chebula* Retz. and reported 100% mortality at 200 ppm (45). The *P. longifolia* methanol extract showed 100% pupal mortality in the present study. The *P. longifolia* leaf extracts showed minimal to no activity against adult mosquitoes, during the susceptibility and repellency activity against *An. stephensi* adult mosquitoes. This shows that, the extracts affect the developing stages of the mosquitoes due to the active phytochemicals present in them. The extracts' effect was further analysed through histological studies and demonstrated morphological and histological changes in larvae, which may be due to the presence of different

groups of phytochemicals that act as mosquitocide.

The leaf extracts of *P. longifolia* contain a wide range of phytochemical constituents, including alkaloids, carbohydrates, starch, glycosides, saponins, steroids, phenols, tannins, flavonoids, proteins, and resins. Katherin *et al*. evaluated the phytochemicals from the *P. longifolia* petroleum ether, ethyl acetate, ethanol, and aqueous extract and reported the presence of flavanoids, triterpenoids, phenolics, tannins, saponins, glycosides, steroids, and reducing sugars. Further study of the GC-MS analysis of the ethyl acetate extract reported the presence of phytol (50). Saha *et al.* studied the antioxidant property of *P. longifolia* bark acetone extract and proved its higher antioxidant activity in comparison to the positive control (38). Zainab *et al*. also evaluated the antioxidant activity of the *P. longifolia* bark extract and found higher antioxidant activity. In the present study, the antioxidant activity of the leaf extract of *P. longifolia* was evaluated, and the study revealed, moderate antioxidant activity. This prove that *P. longifolia* extracts inhibit oxidative damage to the organism.

The multiple compounds present in the extract act as a poison against different developmental stages of *An. stephensi* mosquitoes. This is motivated by the understandable hope that reducing the early stages of mosquitoes will benefit malaria vector control and the safety of public health. Further, the anti-plasmodial activity of the methanol extract revealed minimal activity. Similarly, the NIH/3T3-treated cells also support the non-toxic nature of extracts at lower concentrations. The acute oral toxicity also revealed no toxicity to the tested animal. This will also support the non-toxic nature of the *P. longifolia* methanol extract against non-target species.

Conclusion

The *P. longifolia* leaf extracts have demonstrated efficiency against *An. stephensi*, a species of dangerous mosquito. As a larvicidal, ovicidal, and pupicidal agent, phytochemical components such as phenolics, alkaloids, and terpenoids can kill mosquitoes either collectively or individually. The extracts demonstrated potential antioxidant action and contained considerable amounts of phenolics and flavonoids. This provides important information for creating a novel medical use for *P. longifolia* extracts. The human normal cell lines (NIH/3T3 cells) were not particularly harmful when extracts were used in low quantities and also on non-target species. As a result, *P. longifolia* extracts may be a pioneer in the development of a plant-based mosquitocidal agent against *An. stephensi*, and it may also help with the emergence and spread of vector control in the

future. Further studies are needed to isolate and characterize active components from the *P. longifolia* leaf extracts against malaria-transmitting mosquito *An. stephensi*.

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List of Publications

1. **Sowmyashree, K**., Chalannavar, R. K., Ghosh, S. K., Nityasree, B. R., & Supriya, S. (2019). Effect of Essential Oils of *Aegle marmelos* (L.) Correa and *Psidium guajava* L. On Larvae of Malaria Vector *Anopheles Stephensi* Liston. Life Science, Bioinformatics, Pharmaceutical and Chemical Science. *5*(1), 704-711.
2. **Sowmyashree, K**., Chalannavar, R. K., & Nityasree, B. R. (2023). The efficacy of *Pajanelia longifolia* (Willd.) K. Schum leaf extracts against the malaria vector *Anopheles stephensi* Liston (Diptera: Culicidae). Plant Science Today. 10(2): 316–324.

Conferences and workshop attended

1. **Oral Presentation** on "Effect of essential oils of *Aegle marmelos* (L.) *Correa* & *Psidium gujava* L. on larvae of malaria vector *Anopheles stephensi*" in International conference on Medicinal Plants and Drug Discovery on July 18th - 20th, 2018 in **Bangalore**.
2. **Poster Presentation** on "Larvicidal effect of essential oils from Psidum species against malaria transmitting *Anopheles stephensi* mosquitoes" in International conference in 55th Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine on 13– 14 March 2019 in Kuala Lumpur, **Malaysia**.
3. **Poster Presentation** on "Phytochemical Investigation, In-vitro Antioxidant and Anti-mosquito Activity of *Premna mollissima* Roth" in National Conference organized by Department of Studies in Biochemistry, **Mangalore university**, Post graduate centre, Chikka Aluvara, Kodagu on 6th and 7th October 2017.
4. **Poster Presentation** on "Anti-mosquito Properties of *Sphagneticola trilobata* (L.) Pruski against Malaria Transmitting Mosquito *Anopheles stephensi*" in National Conference organized by **Mangalore university** of Science and technology in association with The Indian Science Congress Association (ISCA) on 8th and 9th September 2017.

Signature of candidate

Signature of research guide